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(54) Title: RAPID ASSAYS FOR THE ASSESSMENT OF ORGAN STATUS BASED ON THE DETECTION OF ONE OR MORE ISOENZYME(S) OF GLUTATHIONE S-TRANSFERASE		
(57) Abstract		
<p>A method for the rapid assessment of organ status, including organ damage following immunological or toxicological insult, in a subject based on the detection of one or more isoenzymes of glutathione S-transferase (GST) in diverse biological fluids comprises contacting a particle-labelled anti-GST antibody specific for said isoenzyme with a sample of a biological fluid suspected of containing said isoenzyme, said antibody having a sensitivity sufficient to detect at least at picomolar amount of said isoenzyme, and capturing the particle-labelled antibody-isoenzyme complex on an immobilised capture antibody to generate a visually detectable signal. The method permits the detection or determination of GST isoenzymes typically in a time of the order of 30 minutes from initial contact with immobilised anti-GST antibody. The method is simple to use and thus can be used, for example, by patients at home to monitor their levels of a particular GST isoenzyme following a liver or renal transplant.</p>		

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DescriptionRapid assays for the assessment of organ status based on the detection of one or more isoenzymes of glutathione S-transferaseTechnical Field

5        This invention relates to a rapid assay format for the detection and/or determination of glutathione S-transferases (GSTs) for use in the assessment of organ status.

Background Art

10      GSTs represent a multigene family of proteins consisting mainly of  $\alpha$ ,  $\mu$ ,  $\theta$ , and  $\pi$ -class isoforms and are responsible for the detoxification of a range of xenobiotics, mainly *via* conjugation to glutathione. Alpha GST ( $\alpha$ GST) is the major hepatic form and is uniformly distributed throughout the liver. In contrast, PiGST ( $\pi$ GST) is located mainly in the epithelial cells lining the bile ducts. A similar

15      unique distribution also exists with respect to the renal situation where  $\alpha$ GST is located in the proximal tubules and  $\pi$ GST is confined to the distal tubule region of the nephron. It can therefore be concluded that measurement of either plasma or urinary GST levels will facilitate the monitoring of either the hepatic or renal status of an individual.

20      Numerous authors have, indeed, used GST measurements to evaluate both liver and renal damage in individuals who have undergone organ transplant or who have suffered organ damage by virtue of exposure to either hepatic or renal toxins (see, for example, Trull, A.K. *et al.*, *Transplantation* (1994) 58, 1345-1351).

25      Currently such measurements are most usually performed using radioimmunoassays (RIA). However, recently a number of microtitre plate-based enzyme immunoassays (EIAs) have become commercially available from Biotrin International Limited, Mount Merrion, County Dublin, Ireland, which allow measurement of  $\alpha$  and  $\pi$ GST levels in biological fluids. These EIAs are now gaining widespread acceptance

among the scientific community despite some initial scepticism (c.f. Beckett, G.J. and Hayes, J.D. (*Advances in Clinical Chemistry* (1993) 30, p.325)). Thus, at present RIA represents the detection method of choice for many workers in the field. Both of these techniques have 5 certain limitations insofar as they require both specialised equipment and highly trained and competent personnel to generate meaningful and accurate results. Such assays are relatively slow requiring on the average 2 hours or more from start to finish. Accordingly, a more rapid assay format for GSTs is required.

10 Methods for the detection of GST isoenzymes are tending to become increasingly complicated as evidenced by the use of Time-Resolved ImmunoFluorometric Assay (TR-IFMA). Tiainen, P *et al.* (*Clin. Chem.* (1996) 42 2, 334-335) compared enzyme immunoassay (EIA) and TR-IFMA for  $\alpha$ GST. The EIA used was Hepkit (trade mark 15 of Biotrin International, Dublin). It was found that the inter-assay precision was better in Hepkit than in TR-IFMA. Also TR-IFMA is stated to have been somewhat more laborious than Hepkit because more replicates (four) and more washing cycles were needed to complete the assay procedure. Thus, it is clear that current research 20 into the detection and quantitation of GST has focussed on the development of more elaborate and complicated detection systems such as TR-IFMA.

Individuals who have undergone organ transplant or who have suffered organ damage by virtue of having been exposed to hepatic or renal toxins must be carefully monitored. This is especially true for 25 individuals who have undergone organ transplant and who undergo a long post-transplant period of therapy with immunosuppressive drugs, in particular cyclosporin. Once such individuals are discharged from hospital they must be continuously monitored, which at present necessitates frequent returns to the relevant hospital unit, so that the 30 requisite GST and other assays can be carried out. Accordingly, it would be most desirable if such persons had a means of self-monitoring which would reduce the number of hospital visits but, more importantly, would give an early indication to the patient and physician

that intervention is required, in the event that an above-normal level of a GST is noted.

Thus, a method which would allow a faster and simpler format for GST detection in plasma, urine and other biological fluids, such as 5 bile, would be a distinct advantage as it would allow the physician to monitor either the hepatic or renal status of an affected individual without recourse to time-consuming or expensive hospital-based tests.

Another area where a rapid assay format is required is in 10 assessing organs prior to transplantation or in *ex vivo* experimentation. Currently, there is no generally acceptable method of donor organ evaluation prior to transplant into the recipient. Consequently it is impossible to accurately predict the final outcome of the transplantation operation as judged by the initial quality of the donor organ. Any 15 method which would contribute to the pre-transplant assessment of the donor organ would greatly aid in the evaluation of the final outcome of the operation and may allow the physician to intervene earlier with post-transplant support therapy to prevent graft loss due to poor quality or host rejection.

As part of normal organ retrieval procedures, the donor organ is 20 equilibrated or perfused with specific reagents (e.g. University of Wisconsin Buffer) once removed from the organ donor. This and other such reagents serve to maintain *ex vivo* organ integrity at low temperatures (4-10°C) prior to insertion into the recipient and are also used to equilibrate the stored organ to body temperature (37°C) prior 25 to surgical reattachment to the recipient. Clearly, the quality of the donor organ will be dependent on many factors such as: donor health; time and temperature of *ex vivo* storage; handling procedures; and the quality of preservation reagents used. Thus, *ex vivo* graft injury, as caused by any of the above factors, could be monitored by evaluation 30 of enzyme leakage from the organ.

Rapid assay formats are known. For example, EP 0 291 194 B1 describes and claims an analytical test device and an analytical method

using said device which are suitable for use in the home, clinic or doctor's surgery and which are indicated to give an analytical result which is rapid and which requires the minimum degree of skill and involvement from the user. The device is indicated to be particularly suitable as a pregnancy testing device. No specific mention is made of GSTs. The levels of marker hormones in pregnant women, such as human chorionic gonadotropin (HCG) tend to be present in higher concentrations, and thus more readily detected, than the levels of GSTs in patients of the type hereinabove described and for whom elevated levels of GST are critical or, indeed, indicative of potential life threatening clinical events.

The difficulty of using enzyme activity measurements for discrimination between the isoforms of GSTs is well documented (Beckett G.J. and Hayes, J.D. (1993) *supra*). As indicated above, other investigators have used RIA (Howie, A.F. *et al.* (*Clinica Chimica Acta* (1989) 184, 269-278) and TR-IFMA (Tiainen, P and Karhi, K.K. *Clin. Chem.* (1994) 40-2, 184-189) to detect GSTs. Each of these techniques is a very sensitive technique for the detection of biomolecules. However, each technique requires specialised equipment and skilled personnel. For example, TR-IFMA requires the use of a special time-resolved fluorometer.

It will be appreciated that a technique is required which is suitable for non-experts or in a crisis situation which is rapid and easy to use. Representative of crisis situations would be liver damage or kidney damage post-transplant or potential liver failure due to paracetamol overdose.

#### Disclosure of Invention

Accordingly, the invention provides a method for the rapid assessment of organ status in a subject based on the detection of one or more isoenzymes of glutathione S-transferase (GST) in diverse biological fluids, which method comprises contacting a particle-labelled anti-GST antibody specific for said isoenzyme with a sample of a

5            biological fluid suspected of containing said isoenzyme, said antibody having a sensitivity sufficient to detect at least a picomolar amount of said isoenzyme, and capturing the particle-labelled antibody-isoenzyme complex on an immobilised capture antibody to generate a visually detectable signal.

By "rapid" herein is meant that the detection or determination of the GST isoenzyme is typically completed in a time of the order of 30 minutes from initial contact with the particle-labelled anti-GST.

10           By organ status herein is included organ damage following immunological or toxicological insult.

15           It will be apparent to one skilled in the art that one of the major advantages of the invention lies in its simplicity, such that it can be used by persons with little or no scientific training to monitor their own state of health and/or to provide rapid information in crisis situations of the type hereinabove described.

20           It will be appreciated that no complicated equipment or detailed laboratory procedures are necessary for assay performance or result evaluation and interpretation. This simplicity of performance and result assessment is significantly at variance with current procedures for GST detection.

Preferably, the GST is  $\alpha$ GST,  $\pi$ GST or  $\mu$ GST of hepatic or renal origin.

Most preferably the GST is  $\alpha$ GST or  $\pi$ GST of hepatic or renal origin.

25           A particular requirement of any rapid assay format for GST is an antibody with the requisite affinity for the GST.

In the case of  $\alpha$ GST, a polyclonal antibody has been raised with an affinity to detect concentrations of  $\alpha$ GST as low as 6.5 ng/ml as

hereinafter described, which contributes greatly to the overall sensitivity of the method.

5 In general, an antibody, which may be monoclonal or polyclonal, with an affinity to detect concentrations of GST isoenzymes as low as 5 ng/ml is preferred for use in accordance with the invention.

The anti-GST antibody bound to the solid phase is suitably polyclonal antibody, more especially polyclonal IgG. However, in certain instances such as in the case of  $\pi$ GST, the solid phase antibody is preferably monoclonal antibody.

10 Suitably, the anti-GST antibody is bound to a nitrocellulose membrane of known type such as that obtainable from Schleicher and Schuell GmbH of Germany. Such membranes have a natural ability to bind proteins, including immunoglobulins. However, it will be appreciated that a variety of solid supports known *per se* can be used as 15 the support for the capture antibody. Such supports are referred to herein generally as the solid phase.

Suitably, the nitrocellulose membrane has a pore size in the range 5-10  $\mu\text{m}$ .

20 Non-specific binding sites on the nitrocellulose membrane are optionally blocked depending on the format of the assay adopted as hereinafter described. Suitably, the blocking of non-specific binding sites can be achieved by treatment with a protein such a bovine serum albumin or milk protein or with a polymer such as polyvinyl-pyrrolidone in a manner known *per se*.

25 The solid phase can have a variety of constructions and can include devices of the type covered EP 0 291 194 B1 referred to above, provided that the anti-GST antibody used has the requisite specificity.

By specificity of the anti-GST antibody as used herein is meant the affinity of the antibody for the relevant GST. This affinity is

expressed in terms of the concentration of analyte detectable rather than in more theoretical terms, such as in terms of a specific affinity  $K_a$  at a given concentration.

5        The solid phase can be accommodated in a housing of a plastics material and can be provided with a wick at one or both ends to facilitate chromatographic movement of the components of the immunoassay.

10      The biological fluid used in the method according to the invention is suitably bile, plasma, serum or urine, more especially plasma, serum or urine.

The biological fluid can be diluted or undiluted (neat).

The biological fluid can also be a perfusate and thus the method according to the invention can be used to evaluate an organ prior to transplantation or *ex vivo* experimentation.

15      As stated above, no such generally accepted method exists for the evaluation of donor organs. We have shown that rapid GST detection in perfusates is possible using the method according to the invention as a marker of *ex vivo* organ status. Rapidity of detection is essential in this situation since the donor organ is undergoing unidirectional and  
20      constant *ex vivo* hypoxia which will ultimately lead to irreversible damage thus rendering the organ useless for transplantation. Therefore, any test which would allow organ assessment will be of great benefit to the physician.

25      The biological fluid is contacted with particle-labelled anti-GST antibody so that the GST isoenzyme to be detected is bound to said particle-labelled anti-GST antibody prior to being contacted with solid phase immobilised antibody. Particles which can be used as labels include colloidal particles, latex particles and metallic sol particles. Metallic sol particles are suitably gold particles having an average diameter in the range 15-40 nm. Such gold particles are commercially

available and are marketed, for example, by Janssen Life Sciences Products.

5           The particle-labelled anti-GST antibody can also be disposed adjacent the sample application end of any device to be contacted with the biological fluid in accordance with the invention.

10          The use of particle-labelled anti-GST antibody means that no amplification step is involved. Thus, in the case of particle-labelled anti-GST antibody detection of GST isoenzymes, detection can be based simply on direct antibody-antigen interaction using particle-labelled antibodies for antigen detection or interaction visualisation with the attendant advantages in the situations hereinabove described.

15          This is all the more surprising, given that it will be apparent from the literature that there has been some scepticism in the scientific community even as regards the use of EIAs for GST detection, even though an amplification system is involved which enhances the sensitivity of detection.

20          The provision of a wick at either end of the solid phase as hereinabove described is particularly preferred when particle-labelled anti-GST IgG is used, since it facilitates chromatographic movement of the labelled IgG. For example, in the case of a nitrocellulose membrane, the provision of a wick at the sample application end and another at the top of the membrane will facilitate chromatographic movement of gold-labelled IgG previously sprayed onto the membrane. Furthermore, the inclusion of purified GST above the immobilised 25       anti-GST IgG on the membrane would act as an internal/procedural control for the gold-labelled anti-GST IgG conjugate function.

30          In this embodiment, liquid present in a biological sample resuspends the gold-labelled anti-GST IgG and any GST isoenzyme present is bound by the gold-labelled IgG. This complex then migrates up or along the membrane attracted by the upper wick. If sufficient GST isoenzyme is present, then the GST/gold-labelled anti-GST IgG

complex is immobilised onto the membrane where bound anti-GST IgG is present. This results in a positive signal indicating that GST is present in the sample. Excess gold-labelled anti-GST IgG conjugate would bind to the procedural control (GST) on the membrane.

5 It will also be appreciated that when particle-labelled antibody is used to bind antigen in the biological sample, then the determination of bound GST can be carried out in a single step by what can be described as a 'one pot' assay.

10 The invention also provides a kit for carrying out a method for the detection of GST as hereinbefore defined which contains an anti-GST antibody which is specific for a GST isoenzyme as herein described.

15 The assay format described herein differs from the aforementioned EIA and RIA type immunoassays in two main respects. Firstly, the order of antigen detection is different insofar as in the method according to the invention the antigen is firstly bound to the particle-labelled antibody (PLA) and then this antigen-PLA complex is captured by the immobilised capture antibody to generate a positive signal. Thus there is no requirement for any intermediate steps such as  
20 membrane washing to remove unbound material (e.g. other urinary, blood or perfusate components) or the addition of any amplification reagent to facilitate detection of bound antigen-PLA complex: the complex is, in fact, 'self-detecting'.

25 This situation contrasts sharply with current methods of GST immunodetection (EIA/RIA) wherein the antigen is firstly contacted with an immobilised capture antibody and after numerous extended washing procedures to remove unbound/interfering components, antibody conjugate (antibody-enzyme or radioactivity labelled antibody) is added to the antigen-antibody complex to detect bound  
30 antigen. In the case where antibody-enzyme conjugates are utilised, it is necessary in all cases to add an additional reagent, a substrate, whereby bound enzyme is detected by the appearance of a coloured product.

Both enzyme- and radioimmunoassay methodologies require expensive and complicated equipment to allow the research scientist or laboratory technician to interpret the significance of the experimental data. When a rapid assay format is utilised for GST immunodetection 5 in accordance with the invention the end result is simply indicated by the presence or absence of a colour (band) and is easily interpretable by the non-expert.

The second main area of difference between the method according to the invention and the known assay systems has already 10 been alluded to above and this aspect centres around the lack of requirement for signal amplification to facilitate GST immunodetection in the present assay format. In fact, the sensitivity of detection required for the rapid detection of GST in accordance with the invention is inherent in the assay format. No extraneous amplification reagents are 15 required other than the particle-labelled antibody and the capture antibody. Other techniques for GST immunodetection all utilise some method of signal amplification including enzymatic production of a coloured product (ELA), fluorescence enhancement using Eu<sup>3+</sup> labelled antibodies (TR-IFMA) and the extensive signal amplification associated 20 with particle emission from the atomic nucleus (i.e., radioactive detection) (RIA).

#### Brief Description of Drawings

Fig. 1 is a schematic representation of a rapid assay format in accordance with the invention using gold-labelled anti-  
25 αGST IgG and membrane bound anti-αGST IgG as described in Examples 2 and 3; and

Fig. 2 is a schematic representation of a rapid assay format in accordance with the invention using gold-labelled anti-  
30 αGST IgG and membrane bound anti-αGST IgG as described in Example 4.

Modes for Carrying Out the Invention

The invention will be further illustrated with reference to the accompanying Examples.

Example 15        $\alpha$ GST purification and antisera generation using purified  $\alpha$ GST $\alpha$ GST purification

Human liver was obtained at autopsy. Liver homogenisation was carried out by adding 113.2 g of liver to 400 ml of a homogenisation buffer at pH 7.2 having the following composition:

10           10 mM sodium phosphate

250 mM sucrose

1 mM EDTA

followed by cell disruption for 2 minutes at 4°C in a Waring (Waring is a trade mark) blender. The resultant homogenate was centrifuged at 11,500 g for 10 minutes after which the supernatant was removed and again centrifuged at 48,000 g for 45 minutes. The final supernatant (380 ml) was dialysed extensively against 20 mM Tris-HCl at pH 7.8 and 4°C. Once dialysis was complete, the dialysate was applied to an S-hexyl GSH Sepharose affinity column.  $\alpha$ GST bound specifically to this column and was eluted using 0.3 mM S-hexyl GSH.

A quality control check was then carried out as follows:

1.  $\alpha$ GST so purified was found to be pure by SDS-PAGE.
2. Purified  $\alpha$ GST reacted with anti- $\alpha$ GST antisera but not with anti- $\pi$  or anti- $\mu$ GST antisera.

3. Isoelectric focussing showed two bands at pI (isoelectric points) 8.5 and 7.8. Purified B<sub>1</sub> B<sub>1</sub> ( $\alpha$ GST) provided one band at pI 8.5. Accordingly, it is postulated that the band with pI 7.8 may be the B<sub>2</sub> containing dimer of  $\alpha$ GST.

5

### Preparation of antisera to human $\alpha$ GST

The preparation of antisera to human  $\alpha$ GST was carried out as follows over the indicated time period using rabbits (internal I.D.: Syncor G and H) using the following protocol.

#### Day 1:

- 10 Carry out a test bleed of 5 ml preserum from the ear of the rabbit then mix 0.5 ml of human  $\alpha$ GST antigen (100  $\mu$ g) with an equal volume of Freund's Complete Adjuvant. Homogenise the antigen and adjuvant to ensure good emulsion. This mixture is then injected subcutaneously into multiple sites on the back of the rabbit which has previously been shaved.  
15

#### Day 28:

- 20 Carry out a test bleed of 5 ml preserum from the ear of the rabbit then mix 0.5 ml of human  $\alpha$ GST antigen (100  $\mu$ g) with an equal volume of Freund's Complete Adjuvant. Homogenise the antigen and adjuvant to ensure good emulsion. This mixture is then injected subcutaneously into multiple sites on the back of the rabbit.

#### Day 42:

A test bleed of 10 ml of blood is taken from the rabbit's ear.

#### Day 56:

- 25 A second boost is given to the rabbit as described on Day 28.

Day 70:

A test bleed of 10 ml of blood is taken from the ear of the rabbit. When the titre is sufficiently high, the rabbit is sacrificed and as much blood as possible is collected.

5      Antibody purification:-

10 ml antisera (rabbit) is applied to a 10 ml column of Protein A Sepharose previously equilibrated in phosphate buffer saline (PBS). Rabbit IgG (total) binds to the Protein A and is eluted using 0.1 M glycine at pH 3.0. Purity is established by SDS-PAGE and functionality (anti-GST activity) is established by microtitre plate coating, dot blot and Western blot analysis and direct conjugation to horseradish peroxidase (HRP).

10

In the case of microtitre plate coating, the concentration of anti- $\alpha$ GST used is 1  $\mu$ g/ml in 0.1 M sodium carbonate buffer at pH 9.6.

15      The anti- $\alpha$ GST IgG-HRP conjugate concentration for use in an enzyme immunoassay for  $\alpha$ GST having an  $\alpha$ GST detection range of 0-50 ng/ml was found to be 77 ng/ml. This result indicates the high sensitivity of the antibody, because normally the concentration required for such enzyme immunoassays (for example, the EIA marketed under the trade mark Mukit by Biotrin International Limited, Mount Merrion, County Dublin, Ireland and EIAs of other companies) would be greater than 700-7,000 ng/ml for other rabbit IgG-HRP conjugates.

20

Example 2 $\alpha$ GST particle-based assay

25      1. Anti- $\alpha$ GST IgG (0.5 mg/ml in PBS) was line-sprayed onto nitrocellulose membrane strips (3 mm x 8 cm). No blocking step was included in the membrane preparation.

However the inclusion of such a procedure may aid strip stability and reduce non-specific binding.

- 5           2. Anti- $\alpha$ GST antibodies were labelled with gold particles (15 and 40 nm diameter) by a contract manufacturer supplied with the antibody and were stored in 0.1% (w/v) sodium azide in 2 mM sodium tetraborate at pH 7.2 as diluent.

#### Assay procedure

- 10           1.  $\alpha$ GST-containing samples were mixed with a given volume of gold-labelled anti- $\alpha$ GST IgG using the following diluent:

15           1% (w/v) bovine serum albumin

1% (w/v) TWEEN-20

in PBS, pH 7.2

15           and were tested as hereinafter described.

- 20           2. The nitrocellulose membrane, pre-coated with anti- $\alpha$ GST IgG (1-3 mm wide), was dipped into the mixture and left in place for up to 3-5 minutes.

#### Testing Procedure

20           The rapid assay procedure was carried out on the following samples (Sample Nos. 1-5):

Sample No 1.  $\alpha$ GST at 25  $\mu$ g/ml (25 mg/l).

25           Sample No 2. Three diluted urine samples with known  $\alpha$ GST concentrations: a) 6.01, b) 18.34 and c) 1.03 ng/ml, respectively- before 1/5 dilution).

Sample No 3. Diluent only.

Sample No 4.  $\pi$ GST.

Sample No 5.  $\alpha$ GST at 50 ng/ml (50  $\mu$ g/L).

Results

5

Sample No 1.

A signal was obtained for the 25  $\mu$ g/ml solution which indicates that even at levels 1000x greater than normal urinary  $\alpha$ GST levels no 'hook effect' (false positive) is observed.

10

Sample No 2a-2c.

$\alpha$ GST present in all three urine samples (diluted 1/5 in dilution buffer) was detected and the signal intensity was proportional to the original  $\alpha$ GST concentration as observed by three independent observers.

15

Sample No 3.

No signal was observed when diluent only was tested in the rapid assay format.

Sample No 4.

No signal was observed.

Sample No 5.

This solution gave a clear detectable signal in the assay.

20

Example 3

$\alpha$ GST particle-based assay

1. Anti- $\alpha$ GST IgG (murine monoclonal; 1mg/ml in PBS) was immobilised onto nitrocellulose membrane strips (0.5 x 4cm)

16

2. The strips were blocked by incubation for 30 minutes at room temperature in a solution of 2% (w/v) non-fat milk and 2% (w/v) polyvinylpyrrolidone in Tris buffered saline, pH 7.4, containing 0.5% (w/v) Tween 20.
- 5 3. The strips were rinsed with distilled water, and dried for 1 hour at room temperature.
4. Anti- $\alpha$ GST antibodies were labelled with gold particles (40nm diameter) by a contract manufacturer supplied with the antibody, and were stored in 0.1% (w/v) sodium azide in 2mM sodium tetraborate at pH 7.2 as diluent.

10

Assay Procedure:

15

1.  $\alpha$ GST containing samples were mixed with a given volume of gold-labelled anti- $\alpha$ GST IgG using Tris buffered saline, pH 7.4, containing 0.5% (w/v) Tween 20 as diluent.
2. A nitrocellulose membrane strip, pre-coated with anti- $\alpha$ GST IgG, was dipped into the mixture and left in place for up to 5 minutes.

20

Testing Procedure:

The rapid assay procedure was carried out on the following samples:

Sample No. 1:  $\alpha$ GST at 250ng/ml.

Sample No. 2: Diluent only.

Results:

25

Sample No. 1: A clear detectable signal was obtained in the assay.

Sample No. 2:      No signal was obtained when diluent only  
                          was tested in the assay.

Principle of the technique and interpretation of results for  
Examples 2 and 3

5       Gold-labelled anti- $\alpha$ GST IgG migrates up the membrane. If  
 $\alpha$ GST is present in the mixture then the gold-labelled IgG/ $\alpha$ GST  
complex binds to the immobilised anti- $\alpha$ GST IgG on the membrane to  
form a pink-red line (precipitate). If no  $\alpha$ GST is present in the  
mixture then the gold-labelled anti- $\alpha$ GST IgG chromatographs to the  
10      top of the membrane and no line (precipitate) is observed.

The technique is also illustrated with reference to Fig. 1. In Fig.  
1 a nitrocellulose membrane strip is indicated generally at 10 and is  
shown dipping into a sample of biological fluid in a receptacle 11  
containing a gold-labelled anti- $\alpha$ GST IgG. A band (1-3 mm) of  
15      immobilised anti- $\alpha$ GST IgG is shown in dotted outline at 12. Direction  
of flow of the sample on the nitrocellulose membrane 10 is indicated by  
the arrow. The gold-labelled anti- $\alpha$ GST IgG binds  $\alpha$ GST, if present,  
and migrates up the membrane and is captured by the immobilised IgG  
at 12. Nitrocellulose membrane 10 is left in contact with the sample  
20      for 3-5 minutes and is then removed as depicted by strips A and B. If  
 $\alpha$ GST is present (positive result) a pink-red line (precipitin line) is  
observed as shown on strip A. If no  $\alpha$ GST is present (negative result)  
no pink-red line forms at 12 as shown on strip B. The arrangement in  
Fig. 1 is purely illustrative and in a commercial embodiment the gold-  
labelled anti- $\alpha$ GST would generally be provided (as a mobile phase) on  
25      the nitrocellulose membrane adjacent the sample application end 13.

The particular advantages of this rapid assay format will be  
apparent, since only a single step is involved in the entire procedure.  
However, it will be appreciated that the rapid assay format according to  
30      the invention represents significant improvement over conventional  
microtitre plated-based immunoassays.

Example 4 $\alpha$ GST particle-based assay

1. Anti- $\alpha$ GST IgG (murine monoclonal; 1mg/ml in PBS) was immobilised onto nitrocellulose membrane strips (0.5 x 4cm) at an area approximately 1cm from the top of the strip.
2. The strips were blocked by incubation for 30 minutes at room temperature in a solution of 2% (w/v) non-fat milk and 2% (w/v) polyvinylpyrrolidone in Tris buffered saline, pH 7.4, containing 0.5% (w/v) Tween 20.
3. The strips were rinsed with distilled water, and dried for 1 hour at room temperature.
4. Anti- $\alpha$ GST antibodies were labelled with gold particles (40nm diameter) by a contract manufacturer supplied with the antibody, and were stored in 0.1% (w/v) sodium azide in 2mM sodium tetraborate at pH 7.2 as diluent.
5. Gold-labelled anti- $\alpha$ GST antibodies were applied to the nitrocellulose membrane strips at an area approximately 3cm from the top of the strip. Prior to application of the gold - labelled antibodies a sublayer of 30% (w/v) sucrose in distilled water was applied to this area of the strip.

Assay Procedure:

1.  $\alpha$ GST containing samples were applied to the nitrocellulose membrane strips.

Testing Procedure:

The rapid assay procedure was carried out on the following samples:

- 5              Sample No. 1:     $\alpha$ GST at 250ng/ml, using Tris buffered saline, pH 7.4, containing 0.5% (w/v) Tween 20 as diluent.
- 10             Sample No. 2:     $\alpha$ GST at 25ng/ml, using Tris buffered saline, pH 7.4, containing 0.5% (w/v) Tween 20 as diluent.
- 15             Sample No. 3:    Diluent only.
- Sample No. 4:    Two diluted perfusate samples with known  $\alpha$ GST concentrations (a) 6716 and (b) 29295 ng/ml, before 1/20 dilution.
- Sample No. 5:    Three diluted serum samples with known  $\alpha$ GST concentrations (a) 26.62 (b) 119.34 and (c) 251.86ng/ml, before 1/20 dilution.
- 20             Sample No. 6:    Three diluted bile samples with known  $\alpha$ GST concentrations (a) 7.26 (b) 31.93 and (c) 68.12 ng/ml, before 1/20 dilution.
- 25             Sample No. 7:    A diluted urine sample with a known  $\alpha$ GST concentration of 88.07ng/ml before 1/20 dilution.

Results:

- 25             Sample No. 1:    A clear detectable signal was obtained in the assay.

- Sample No. 2: A clear detectable signal was obtained in the assay.
- Sample No. 3: No signal was obtained when diluent only was tested in the assay.
- 5              Sample No. 4:  $\alpha$ GST present in both perfusate samples was detected.
- 10             Sample No. 5: No signal was obtained when the serum sample containing 26.62ng/ml  $\alpha$ GST (before 1/20 dilution) was tested, indicating that the assay does not detect  $\alpha$ GST at levels corresponding to normal serum levels.  $\alpha$ GST present in the samples containing 119.34 and 251.86 ng/ml  $\alpha$ GST (before 1/20 dilution) was detected.
- 15             Sample No. 6: No signal was obtained when the bile samples containing 7.26 and 31.93ng/ml  $\alpha$ GST (before 1/20 dilution) were tested, indicating that the assay does not detect  $\alpha$ GST at these levels.
- 20             Sample No. 7:  $\alpha$ GST present in the sample containing 68.12ng/ml  $\alpha$ GST (before 1/20 dilution) was detected.
- 25             Principle of the technique and interpretation of results for Example 4

As the test sample migrates along the nitrocellulose membrane strip it mixes with the gold-labelled anti- $\alpha$ GST antibodies on the strip.

If  $\alpha$ GST is present in the sample, the labelled anti- $\alpha$ GST antibodies bind to the  $\alpha$ GST forming an antibody-antigen complex. This complex migrates along the strip and binds to the anti- $\alpha$ GST IgG immobilised onto the strip, and produces a pink-red line. If no  $\alpha$ GST is present in the sample the labelled anti- $\alpha$ GST antibodies migrate to the top of the strip, and no line is observed.

The technique is also illustrated schematically with reference to Fig 2. A nitrocellulose membrane strip is shown generally at 20; near its end 21 is an area 22 bearing gold-labelled anti- $\alpha$ GST antibodies. Upstream of the strip 20 is an area 23 with immobilised anti- $\alpha$ GST IgG in the form of a band. Sample is applied to the end 21, and migrates along the strip towards the end 24. In so doing, the sample mixes with the gold-labelled anti- $\alpha$ GST antibodies at 22 and  $\alpha$ GST, if present in the sample, binds to the gold-labelled antibodies forming an antibody - antigen complex. This complex binds to the anti- $\alpha$ GST IgG immobilised at 23 and produces a pink-red line. If no  $\alpha$ GST is present in the sample, no pink-red line forms at 23.

#### Example 5

##### $\pi$ GST particle-based assay

- 20        1. Anti- $\pi$ GST IgG (murine monoclonal; 0.6 mg/ml in PBS) was spotted onto nitrocellulose membrane strips (1  $\mu$ l/dot  $\equiv$  0.6  $\mu$ g). No blocking step was included in the membrane preparation, however the inclusion of such a procedure may aid strip stability and reduce non-specific binding.
- 25        2. Anti- $\pi$ GST antibodies (rabbit polyclonal) were labelled with gold particles (40 nm diameter) by a contract manufacturer supplied with the antibody and were stored in 0.1% (w/v) sodium azide in 2 mM sodium tetraborate at pH 7.2 as diluent.

Assay procedure

1. πGST-containing samples (10μl each @ 1-500 μg/ml) were mixed with a given volume (50 μl) of gold-labelled anti-πGST IgG and 140 μl of the following diluent:

5                   1% (w/v) bovine serum albumin  
                  1% (w/v) TWEEN-20  
                  in PBS, pH 7.2

and were tested as hereinafter described.

2. The nitrocellulose membrane, pre-coated with anti-πGST IgG (0.6 μg/dot), was dipped into the mixture and left in place for up to 3-15 minutes.

Testing Procedure

The rapid assay procedure was carried out on the following samples:

15                 Sample Nos. 1-4: containing πGST in buffer only;

                       Sample No. 5: containing buffer only; and

                       Sample Nos. 6-15: urine, bile and plasma containing πGST, as set forth in Table 1.

20                 Sample Nos. 7 and 8 (urine) and 12 and 13 (plasma) were spiked with purified πGST. However the bile samples contained inate πGST.

Table 1

Sample No.	Initial conc. (ng/ml)	Final conc. (ng/ml)	Amount Present (ng)
1	500,000	25,000	5000
2	50,000	2,500	500
3	5,000	250	50
4	1,000	50	10
5	0	0	0
6 (Urine)	30	1.5	0.3
7 (Urine)	1000	50	10
8 (Urine)	5000	250	50
9 (Bile)	150	7.5	1.5
10 (Bile)	>5000	>250	>50
11 (Bile)	3730	186.5	37.2
12 (Plasma)	1000	50	10
13 (Plasma)	5000	250	50
14 (Plasma)*	1000	250	50
15 (Plasma)	120	6	1.2

\* (50 µl sample + 50 µl gold-labelled anti-πGST IgG and 100 µl diluent)

Results

The results are set forth in Table 2

Table 2

Sample No.	Result
Sample No. 1	YES
Sample No. 2	YES
Sample No. 3	YES
Sample No. 4	YES
Sample No. 5	NO
Sample No. 6	NO
Sample No. 7	YES
Sample No. 8	YES
Sample No. 9	NO
Sample No. 10	YES
Sample No. 11	YES
Sample No. 12	YES
Sample No. 13	YES
Sample No. 14	YES
Sample No. 15	NO

5            It can be seen from the above results that  $\pi$ GST is detectable in urine, bile and plasma samples. The sensitivity of the assay appears to be around 50 ng/ml but it is likely that assay optimisation could improve this level of sensitivity. However, this level of  $\pi$ GST has already been observed in biological samples (e.g. bile and tissue  
10          perfusion media).

A monoclonal-polyclonal antibody sandwich was found to be necessary for  $\pi$ GST detection by this assay format. However it is possible that if a polyclonal antibody of sufficient sensitivity was identified that this IgG would function in the assay (as in the case of 5 rapid assay format described hereinabove for  $\alpha$ GST). Alternatively, a monoclonal/monoclonal type assay may also give sufficient sensitivity.

Example 6

$\pi$ GST particle-based assay

1. Anti- $\pi$ GST IgG (murine monoclonal; 0.6mg/ml in PBS) 10 was immobilised onto nitrocellulose membrane strips (0.5 x 4cm)
2. The strips were blocked by incubation for 30 minutes at room temperature in a solution of 2% (w/v) non-fat milk and 2% (w/v) polyvinylpyrrolidone in Tris buffered saline, pH 7.4, containing 0.5% (w/v) Tween 20. 15
3. The strips were rinsed with distilled water, and dried for 1 hour at room temperature.
4. Anti- $\pi$ GST antibodies were labelled with gold particles (40nm diameter) by a contract manufacturer supplied with 20 the antibody, and were stored in 0.1% (w/v) sodium azide in 2mM sodium tetraborate at pH 7.2 as diluent.

Assay Procedure:

1.  $\pi$ GST containing samples were mixed with a given volume of gold-labelled anti- $\pi$ GST IgG using Tris buffered saline, pH 7.4, containing 0.5% (w/v) Tween 20 as diluent. 25

2. A nitrocellulose membrane strip, pre-coated with anti- $\pi$ GST IgG, was dipped into the mixture and left in place for up to 30 minutes.

Testing Procedure:

5 The rapid assay procedure was carried out on the following samples:

Sample No. 1:  $\pi$ GST at 250ng/ml.

Sample No. 2: Diluent only.

Results:

10 Sample No. 1: A clear detectable signal was obtained in the assay.

Sample No. 2: No signal was obtained when diluent only was tested in the assay.

Principle of the technique and interpretation of results for Examples 5 and 6

15 Gold labelled anti- $\pi$ GST IgG migrates up the membrane. If  $\pi$ GST is present in the mixture then the gold-labelled IgG/ $\pi$ GST complex binds to the immobilised anti  $\pi$ GST monoclonal IgG on the membrane to form a pink-red line (precipitate). If no  $\pi$ GST is present in the mixture then the gold-labelled anti- $\pi$ GST IgG chromatographs to the top of the membrane and no line (precipitate) is observed.

20 It will be appreciated that this assay format also represents a significant step forward when compared to conventional microtitre plate (or membrane)-based assays, since only a single step is involved in the entire procedure.

Example 7 $\pi$ GST particle-based assay

1. Anti- $\pi$ GST IgG (murine monoclonal; 0.6mg/ml in PBS) was immobilised onto nitrocellulose membrane strips (0.5 x 4cm) at an area approximately 1cm from the top of the strip.
2. The strips were blocked by incubation for 30 minutes at room temperature in a solution of 2% (w/v) non-fat milk and 2% (w/v) polyvinylpyrrolidone in Tris buffered saline, pH 7.4, containing 0.5% (w/v) Tween 20.
3. The strips were rinsed with distilled water, and dried for 1 hour at room temperature.
4. Anti- $\pi$ GST antibodies were labelled with gold particles (40nm diameter) by a contract manufacturer supplied with the antibody, and were stored in 0.1% (w/v) sodium azide in 2mM sodium tetraborate at pH 7.2 as diluent.
5. Gold-labelled anti- $\pi$ GST antibodies were applied to the nitrocellulose membrane strips at an area approximately 3cm from the top of the strip. Prior to application of the gold-labelled antibodies a sublayer of 30% (w/v) sucrose in distilled water was applied to this area of the strip.

Assay Procedure:

1.  $\pi$ GST containing samples were applied to the nitrocellulose membrane strips.

Testing Procedure:

The rapid assay procedure was carried out on the following samples:

5              Sample No. 1:     $\pi$ GST at 250ng/ml, using Tris buffered saline, pH 7.4, containing 0.5% (w/v) Tween 20 as diluent.

10             Sample No. 2:     $\pi$ GST at 25ng/ml, using Tris buffered saline, pH 7.4, containing 0.5% (w/v) Tween 20 as diluent.

15             Sample No. 3:    Diluent only.

Results:

15             Sample No. 1 and 2:    A clear detectable signal was obtained in the assay for both samples, and the signal intensity was proportional to the  $\pi$ GST concentration.

20             Sample No. 3:    No signal was obtained when diluent only was tested in the assay.

Principle of the technique and interpretation of results for Example 7

20             As the test sample migrates along the nitrocellulose membrane strip it mixes with the gold-labelled anti- $\pi$ GST antibodies on the strip. If  $\pi$ GST is present in the sample, the labelled anti- $\pi$ GST antibodies bind to the  $\pi$ GST forming an antibody-antigen complex. This complex binds to the anti- $\pi$ GST IgG immobilised onto the strip, and produces a pink-red line. If no  $\pi$ GST is present in the sample the labelled anti- $\pi$ GST antibodies migrate to the top of the strip, and no line is

observed. Thus, the technique corresponds to that described in relation to Example 4 and as illustrated in Fig. 2.

Claims:-

1. A method for the rapid assessment of organ status in a subject based on the detection of one or more isoenzymes of glutathione S-transferase (GST) in diverse biological fluids, which method  
5 comprises contacting a particle-labelled anti-GST antibody specific for said isoenzyme with a sample of a biological fluid suspected of containing said isoenzyme, said antibody having a sensitivity sufficient to detect at least a picomolar amount of said isoenzyme, and capturing the particle-labelled antibody-isoenzyme complex on an immobilised  
10 capture antibody to generate a visually detectable signal.
2. A method according to Claim 1, wherein the GST is alpha GST ( $\alpha$ GST).
3. A method according to Claim 1, wherein the GST is piGST ( $\pi$ GST).
- 15 4. A method according to any one of Claims 1-3, wherein the anti-GST antibody is polyclonal antibody.
5. A method according to Claim 4, wherein the anti-GST antibody is anti- $\alpha$ GST antibody which has an affinity to detect  $\alpha$ -GST concentrations of as low as 6.5 ng/ml.
- 20 6. A method according to any preceding claim, wherein the capture antibody is bound to a nitrocellulose membrane.
7. A method according to Claim 6, wherein non-specific binding sites present on the nitrocellulose membrane are blocked.
- 25 8. A method according to any preceding claim, wherein the biological fluid is diluted.
9. A method according to any preceding claim, wherein the biological fluid is selected from bile, plasma, serum and urine.

10. A method according to any one of Claims 1-8, wherein the biological fluid is a perfusate and the method is used to evaluate an organ prior to transplantation or *ex vivo* experimentation.

5 11. A method according to any preceding claim, wherein said particles are gold particles having an average diameter in the range 15-40 nm.

12. A kit for carrying out a method according to any one of Claims 1-11, which contains particle-labelled anti-GST antibody which is specific for a GST isoenzyme.

1/1

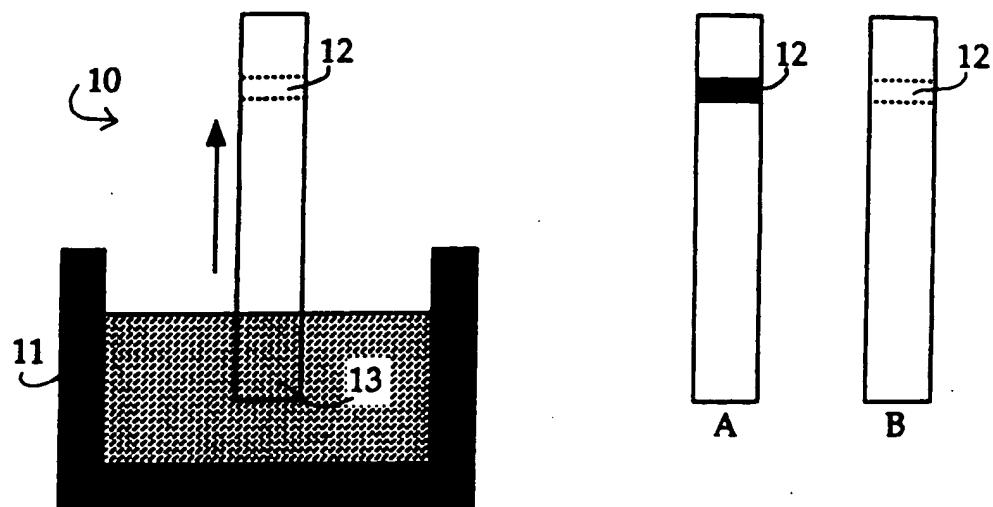


Fig. 1

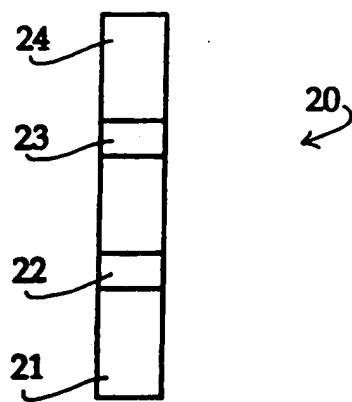


Fig. 2

## INTERNATIONAL SEARCH REPORT

International Application No  
PCT/IE 96/00019

A. CLASSIFICATION OF SUBJECT MATTER  
IPC 6 G01N33/573 G01N33/558 G01N33/548 G01N33/58

According to International Patent Classification (IPC) or to both national classification and IPC

## B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)  
IPC 6 G01N

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

## C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	WO,A,93 22452 (C. G. KILTY ET AL.) 11 November 1993 see the whole document ---	1-12
Y	EP,A,0 560 410 (UNILEVER N. V.) 15 September 1993 see page 11, line 1-56 see page 13, line 10-23 & EP,A,0 291 194 cited in the application ---	1-12
Y	WO,A,90 12088 (THE UNITED STATES OF AMERICA) 18 October 1990 see page 11, line 7 - line 28 -----	3

Further documents are listed in the continuation of box C.

Patent family members are listed in annex.

## \* Special categories of cited documents :

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1 Date of the actual completion of the international search	Date of mailing of the international search report
8 August 1996	14.08.96

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## INTERNATIONAL SEARCH REPORT

Information on patent family members

International Application No
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Patent document cited in search report	Publication date	Patent family member(s)		Publication date
WO-A-9322452	11-11-93	EP-A-	0640145	01-03-95
		JP-T-	7506428	13-07-95
		US-A-	5217868	08-06-93
-----				
EP-A-560410	15-09-93	AU-B-	626207	23-07-92
		AU-B-	1622888	02-12-88
		AU-B-	8048994	09-03-95
		AU-B-	8049094	09-03-95
		DE-D-	3887771	24-03-94
		DE-T-	3887771	26-05-94
		DE-U-	8805565	18-08-88
		EP-A-	0291194	17-11-88
		EP-A-	0560411	15-09-93
		ES-T-	2050704	01-06-94
		FR-A-	2614423	28-10-88
		WO-A-	8808534	03-11-88
		GB-A-	2204398	09-11-88
		HK-A-	140995	15-09-95
		JP-A-	6180320	28-06-94
		JP-A-	6160388	07-06-94
		JP-B-	7046107	17-05-95
		JP-T-	1503174	26-10-89
		AU-B-	656966	23-02-95
		AU-B-	1704992	27-08-92
		AU-B-	656967	23-02-95
		AU-B-	1705092	27-08-92
-----				
WO-A-9012088	18-10-90	AU-B-	5435990	05-11-90
-----				